REMARKS

After the above amendments, Claims 46-66, 72-77, 81 and 186-266 are pending.

Support for the amendment to Claim 46 with respect to the degree of dephosphorylation may be found at, *e.g.*, page 7, lines 11-20, and Examples 1-3 of the specification. As noted there, proteins and peptides which are useful in the practice of the invention must be "at least partially dephosphorylated." Proteins and peptides which are "at least partially dephosphorylated" must have the number of phosphorylated amino acids present in the population of proteins or peptides reduced by at least 10% (*i.e.*, they must be at least 10% dephosphorylated).

A. Restriction Requirement

In response to the Examiner's restriction requirement, Applicant elected the Group 3 claims (Claims 46-75 and 79-81) without traverse. In response to the Examiner's requirement of an "Additional Election" as described at page 5 of the Office Action dated March 27, 2006, Applicant elected phosvitin with traverse. Applicant continues to traverse this requirement for an Additional Election for the reasons of record and for the following additional reasons.

As noted in Applicant's previous response to the Additional Election requirement, Applicant's compounds all fall within the Markush group of phosphate acceptor compounds (PACs, IPACs, or EPACs) that have common features. Specifically, they are phosphate accepting molecules and are useful in the treatment of diseases mediated by the increased phosphorylation of proteins and peptides by kinases. See page 4, lines 1-8 and lines 13-15, of the present application.

With respect to claims covering Markush groups, a species election, rather than the Examiner's requirement of an Additional Election of an invention, is appropriate. See MPEP § 803.02 (Rev. 5, Aug. 2006). In particular in this section 803.02 of the MPEP, on page 800-5, it states:

> A Markush-type claim may include independent and distinct inventions. This is true where two or more of the members are so unrelated and diverse that a prior art reference anticipating the claim with respect to one of the members would not render the claim obvious under 35 U.S.C. 103 with respect to the other member(s). In applications containing a Markush-type claim that encompasses at least two independent or distinct inventions, the examiner may require a provisional election of a single **species** prior to the examination on the merits. . . . Following election, the Markush-type claim will be examined fully with respect to the elected **species** and further to the extent necessary to determine patentability. [Emphasis added.]

Accordingly, the Examiner is respectfully requested to revise the Additional Election requirement to make it an election of a species, instead of the election of an invention.

B. Objections To The Specification

1. Objection to page 64, lines 11 and 19 of the Specification

The Examiner has objected to page 64, line 11, of the specification on the basis that "BSA" and "ELIS" should be set forth in full the first time they are used. This has already been done in the specification for BSA. Immediately before the abbreviation is used, the full term (bovine serum albumin) is stated (see page 64, line 11). "ELIS" is a grade of BSA, and it is also defined in the specification immediately after the term (see page 64, line 11). Thus, the Examiner is requested to withdraw this objection.

The Examiner has objected to page 64, line 19, of the specification on the basis that "ELISA" should be set forth in full the first time it is used. By the above amendments of the specification, this has been done. Thus, this objection is overcome.

2. Objection to page 6, line 2, page 9, line 6, page 12, line 12, and page 14, line 28 of the Specification

The Examiner has objected to page 6, line 2, page 9, line 6, page 12, line 12, and page 14, line 28, of the specification on the basis that they contained embedded hyperlinks. By the above

amendments of the specification, these hyperlinks have been deleted in the manner advised by the Examiner. Thus, this objection is overcome.

3. Objection to Claim 80

The Examiner has objected to Claim 80 on the basis that EPACs should be set forth in full the first time it is used. By the above amendments of the claims, Claim 80 has been canceled. Thus, this objection is moot.

C. Section 112 Rejections

The Examiner has rejected Claims 67, 76, 81 and 185 on the basis that they are indefinite. Applicant respectfully traverses these rejections for the following reasons and asks that they be withdrawn.

It is the Examiner's position that Claims 67 and 185 are indefinite because of the phrase "attached to" used in those claims. It is the Examiner's position that it is unclear whether "attached to" refers to covalent or noncovalent linkages. The attachment of the PACs to targeting molecules is illustrated at page 15, line 8, through page 17, line 22, of the present application. As described there, the targeting molecules can be covalently or noncovalently attached to a PAC. Thus, it is submitted that the meaning of "attached to" is clear. The passage on page 48, lines 9-13, referenced by the Examiner does not apply to the attachment of a targeting molecule to a PAC.

It is the Examiner's position that Claim 76 is indefinite because the phrase "random sequence" used in that claim is not defined in the specification. However, the meaning of "random sequence" is provided on page 12, lines 5-18. As set forth there, it means that a protein or peptide, or a portion of a protein or peptide, containing a phosphorylatable amino acid need not contain a phosphorylation site (*i.e.*, the sequence around the phosphorylatable amino acid can be random). Accordingly, it is submitted that the meaning of this term is also clear.

It is the Examiner's position that Claim 81 is indefinite because the phrases "a combination of EPACs" and "other EPACs" used in the claim are drawn to non-elected inventions. Applicants submit that these phrases are clear (see the discussion of EPACs on pages 5-12 of the application), and that this is not a section 112 issue.

D. Section 102 Rejections

1. Rejection of Claims 46-49, 62, 67-70, 72, 74, 79 and 185 as anticipated by GB 1,350,197

The Examiner has rejected Claims 46-49, 62, 67-70, 72, 74, 79 and 185 as anticipated by GB 1,350,197 (Istituo Farmacologico Serono or IFS) as evidenced by Fujino et al., *Gamete Res.*, 7:249-257 (1983). Applicant respectfully traverses this rejection.

The Examiner cites IFS as teaching pharmaceutical compositions comprising phosvitin. IFS does teach certain pharmaceutical compositions comprising phosvitin, but IFS teaches that the phosvitin used in these compositions is phosvitin as it is obtained from egg yolks without any dephosphorylation. Thus, IFS alone does not anticipate the rejected claims.

The Examiner cites Fujino et al. as teaching that naturally-occurring phosvitin has certain inherent properties. In particular, the Examiner contends that Fujino et al. teaches that naturally-occurring phosvitin is not fully phosphorylated (*i.e.*, it is partially desphosphorylated) and is, therefore, capable of acting as a phosphate acceptor.

To rely on inherency, the Examiner must establish that the phosvitin used in the IFS compositions necessarily has these properties. Inherency cannot be established by speculation or possibilities.

Contrary to the Examiner's contentions, the teachings of Fujino et al. do not establish that phosvitin is necessarily at least partially dephosphorylated.[1]

First, the phosvitin used in the experiments described in Fujino et al. appears to have been partially dephosphorylated phosvitin, not naturally-occurring phosvitin, as contended by the Examiner. See the section entitled "Chemicals" on page 251 of Fujino et al.

Second, the sea urchin kinase used by Fujino et al. was only partially purified (see, *e.g.*, the Abstract of Fujino ct al.). The use of such impure kinase preparations can lead to incorrect

[1] By making these arguments, Applicant does not concede that Fujino et al. is being properly used as a second reference in this section 102 rejection.

interpretations of results. *See, e.g.*, Gill and Walton, *Advances in Cyclic Nucleotide Research*, **10**:93-106 (1979), cited in Fujino et al. (copy being submitted herewith as Appendix A). For instance, no measurement was made of phosphatase activity in these impure materials. A phosphatase, if present, might have dephosphorylated the added phosvitin, which could then have been re-phosphorylated with the radioactively labeled phosphate by the kinase.

Third, the sea urchin kinase itself was shown by Fujino et al. to remove phosphate from phosvitin. See the final paragraph of the Results section, paragraph bridging pages 254-255, and the penultimate paragraph of the Discussion section, page 256, of Fujino et al. Thus, the sea urchin kinase may have dephosphorylated the phosvitin and then re-phosphorylated it with the radioactively labeled phosphate.

Thus, contrary to the Examiner's contentions, Fujino et al. does not establish that the phosvitin used in the pharmaceutical compositions of IFS has the inherent characteristic of not being fully phosphorylated (*i.e.*, being partially dephosphorylated), as alleged by the Examiner, and IFS does not anticipate any of the rejected claims.

Finally, the phrase "at least partially dephosphorylated" is defined in the present application to mean at least 10% dephosphorylated. See page 7, lines 11-20, of the present application and the above remarks. Even assuming that the Examiner's contentions are correct that naturally-occurring phosvitin is not fully dephosphorylated, there is no teaching or suggestion in Fujino et al. that the phosvitin used by them was at least 10% dephosphorylated.[2]

Applicants wish to make other points about the Examiner's contentions:

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[2] The phosvitin used by Fujino et al. was obtained from Sigma. Applicant has shown that naturally-occurring phosvitin from Sigma which was not dephosphorylated is not suitable for use in the present invention. See Example 1 of the present application. Assuming the phosvitin used by Fujino et al. was naturally-occurring phosvitin from Sigma which was not dephosphorylated, as contended by the Examiner, Example 1 provides evidence that such phosvitin is not suitable for use in the present invention.

First, iron is not a targeting molecule, since it will not direct a PAC to which it is bound to a selected cell, tissue or organ. See page 15, line 9 through page 17, line 22, of the present application.

Second, intramuscular administration (*i.e.*, administration into the muscle) is not topical administration. Topical administration means administration on the surface of, *e.g.*, the skin, mucous membranes, lungs or eyes. IFS does not teach or suggest any pharmaceutical compositions for topical administration, since it teaches that phosvitin is to be used to treat heart diseases. See page 2, lines 33-40, of IFS.

Third, pharmaceutical compositions suitable for topical administration have different formulations (compositions) than other pharmaceutical compositions. See page 22, line 26, through page 30, line 8, of the present application. Similarly, pharmaceutical compositions suitable for topical administration to the skin have different formulations (compositions) than other pharmaceutical compositions, including other topical pharmaceutical compositions. See page 22, line 26, through page 30, line 8, of the present application. Thus, "suitable for topical administration" and "suitable for topical administration to the skin" are used in the claims to mean a difference in composition. This has now been made clearer by changing "suitable for topical administration" to "formulated for topical administration" in the relevant claims.

Finally, EPACs and IPACs are intended for different uses, but also have different physical characteristics. See, *e.g.*, page 5, lines 5-11, page 6, lines 6-9, page 12, lines 21-24, and page 13, lines 11-16, of the present application.

2. Rejection of Claims 46-49, 62, 67-70, 72, 74, 79 and 185 as anticipated by U.S. Patent No. 3,966,915

The Examiner has rejected Claims 46-49, 62, 67-70, 72, 74, 79 and 185 as anticipated by U.S. Patent No. 3,966,915 (Caprino) as evidenced by Fujino et al., *Gamete Res.*, 7:249-257 (1983). Applicant respectfully traverses this rejection for the same reasons as discussed above for traversing the rejection based on IFS and Fujino et al. Accordingly, Applicant also asks that this rejection be withdrawn.

3. Rejection of Claims 46-53, 72, 74 and 79-80 as anticipated by Jiang et al.

The Examiner has rejected Claims 46-53, 72, 74 and 79-80 as anticipated by Jiang et al., *J. Agric. Food Chem.*, **48**:990-994 (2000). It is the Examiner's position that Jiang et al. teaches an aqueous solution containing dephosphorylated phosvitin that is considered to be a pharmaceutical composition.

The claims have been amended to specify that the claimed pharmaceutical compositions are not aqueous solutions. Thus, this rejection is overcome.

4. Rejection of Claim 81 as anticipated by Pierce

The Examiner has rejected Claim 81 as anticipated by Pierce, *Instructions for Gel Code® Phosphoprotein Staining Kit*, pages 1-3 (2001). Applicant respectfully traverses this rejection.

It is the Examiner's position that Pierce teaches a kit comprising a container holding phosvitin. However, the phosvitin is the positive control (see page 1 of Pierce) and, therefore, would not have been dephosphorylation (see Table 1, page 3 of Pierce). Thus, Pierce does not anticipate Claim 81, and the Examiner is requested to withdraw this rejection.

E. Section 103 Rejection

The Examiner has rejected Claims 46-47, 49, 62, 71-72, 74 and 79 as obvious over U.S. Patent No. 6,569,839 (McKay) in view of Kipping et al., *Biochemistry,* **40**:7957-7963 (2001). It is the Examiner's position that McKay teaches a pharmaceutical composition comprising phosvitin and hirudin, a plasma protein. Applicant respectfully traverses this rejection.

There is no teaching or suggestion in McKay that the phosvitin used in the compositions of McKay is dephosphorylated. Indeed, to function as a anticoagulant, the phosvitin would have to be phosphorylated. See, *e.g.*, Church et al., *FEBS Letters*, **237**:26-30 (1988), particularly the second and last paragraphs of the Discussion section (copy being submitted herewith as Appendix B). Thus, McKay would not have taught or suggested pharmaceutical compositions comprising

dephosphorylated phosvitin to those skilled in the art or motivated those skilled in the art to make such pharmaceutical compositions.

It is also the Examiner's position that the pharmaceutical composition of McKay can be an emulsion, such as a cream. However, this is incorrect. The passage referred to by the Examiner is a discussion of the preparation of a solution. McKay does teach that some of the components used in the preparation of the solution may be in suspension or in an emulsion, if they cannot be dissolved in the solution carrier (column 10, lines 45-50, of McKay). McKay does not specify which components these might be, but phosvitin is very hydrophilic (with over 100 phosphate groups), and would be expected to be highly soluble in aqueous solutions.

To the extent that the Examiner may be relying on the teachings of Fujino et al. as teaching that the phosvitin taught by McKay is inherently at least partially dephosphorylated or is inherently a kinase substrate, he should expressly state that he is doing so and explain how the Fujino et al. teachings apply to the phosvitin used by McKay.

The teachings of Kipping et al. add nothing to those of McKay. The Examiner relies on Kipping et al. for teaching that residue Thr^{45} of hirudin is phosphorylatable, but this does not provide any information on the phosphorylation status of the hirudin used in the compositions of McKay.

It should also be noted that hirudin is not a plasma protein, as contended by the Examiner. It is an anticoagulant protein originally isolated from the saliva of leeches. See, *e.g.*, *PDR Medical Dictionary*, page 799 (1st ed., 1995) (copy being submitted herewith as Appendix C).

Finally, as noted above, EPACs and IPACs are intended for different uses, but also have different physical characteristics. See, *e.g.*, page 5, lines 5-11, page 6, lines 6-9, page 12, lines 21-24, and page 13, lines 11-16, of the present application. McKay does not teach or suggest using anything smaller than full-length phosvitin.

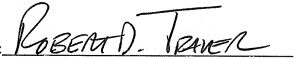
For all of the foregoing reasons, the combined teachings of McKay and Kipping et al. would not have made the claimed invention obvious, and the Examiner is requested to withdraw this rejection.

CONCLUSION

Applicant believes that all pending claims are in condition for allowance and such disposition is respectfully requested. In the event that a telephone conversation would further prosecution and/or expedite allowance, the Examiner is invited to contact the undersigned.

Respectfully submitted,

SHERIDAN ROSS P.C.

By: 

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Date: November 15, 2006

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Assay of Cyclic Nucleotide-Dependent Protein Kinases

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I. PRINCIPLE

Both cAMP- and cGMP-dependent protein kinase (protein kinase A and protein kinase G) catalyze the transfer of the γ-phosphate of ATP to serine residues of an appropriate substrate.

$$\text{Protein} + \text{ATP} \rightleftharpoons \text{phosphoprotein} + \text{ADP} \qquad (1)$$

cAMP-dependent protein kinase is a tetramer composed of regulatory cAMP binding and catalytic phosphotransferase subunits (1–3). Holoenzyme activation by cAMP involves dissociation of a regulatory dimer from two catalytic subunits.

$$\underset{\text{inactive}}{R_2C_2} + 2\text{cAMP} \rightleftharpoons \underset{\text{active}}{R_2 \cdot \text{cAMP}_2} + 2C \qquad (2)$$

where R refers to the regulatory cyclic nucleotide binding subunit and C refers to the catalytic phosphotransferase subunit.

cGMP-dependent protein kinase is a dimer composed of two identical subunits covalently linked by disulfide bonds (4–6). Holoenzyme activation by cGMP appears to involve alterations in enzyme conformation through

allosteric interactions because dissociation does not occur upon cGMP binding:

$$(RC)_2 + 2cGMP \rightleftharpoons (RC)_2 \cdot cGMP_2 \quad (3)$$

Enzyme activity is measured by the rate of incorporation of radioactive phosphate from [γ-^{32}P]ATP into acid-insoluble protein under conditions where activity is dependent on the presence of the cyclic nucleotide. The separation of phosphoprotein from ATP is accomplished by acid precipitation of protein onto filter paper disks. When required, activity can be measured by changes in the activity of interconvertible enzymes which are functional substrates for cyclic nucleotide-dependent protein kinases. Both cyclic nucleotide-dependent protein kinases specifically use ATP as phosphate donor, are preferentially responsive to their respective cyclic nucleotides, and phosphorylate a variety of protein substrates (e.g., histone, protamine, casein, phosphorylase b kinase, glycogen synthetase, hormone-sensitive lipase, cardiac muscle troponin, fructose-1,6-diphosphatase, pyruvate kinase, membrane proteins, etc.) (7–11). Histone has been most widely used as a general substrate for cyclic nucleotide-dependent protein kinases.

In addition to kinase activity, the binding specificity of each enzyme for its respective cyclic nucleotide provides an alternate assay. Direct binding of radiolabeled cyclic nucleotide and subsequent separation of the cyclic nucleotide-protein complex from free nucleotide by retention on Millipore filters provides a convenient and rapid assay.

II. MATERIALS

1. *Kinase assay.* The kinase assay requires the following materials:

*Test tubes (6 × 50 mm) and test tube rack
*Pipets, microliter (Lang-Levy)
Paper filter disks (Whatman 3MM, 2.4 cm)
Pins (stainless steel)
Styrofoam board (packing material)
*Water baths, 30 and 90°C
*Pipets (Pasteur)
Infrared lamp
*Counting vials (22-mm neck)
*Scintillation counter

2. *Binding assay.* In addition to starred items above, the binding assay requires the following items:

Millipore filters (25 mm, 0.45 μm)
Filter apparatus, an apparatus with 10 filter spaces is convenient (Hoefer Scientific).
Reagent bottle, dispensing (5 ml)

III. REAGENTS

1. *Kinase assay.* The kinase assay requires the following reagents:

*Potassium phosphate, monobasic (KH_2PO_4)
*Potassium phosphate, dibasic ($K_2HPO_4 \cdot 3\ H_2O$)
*Magnesium chloride ($MgCl_2 \cdot 6\ H_2O$)
*Dithiothreitol (DTT)
Histone H2b (type VII, Sigma Chemical Co.)
Adenosine 5′-triphosphate, dipotassium salt (ATP)
Adenosine 5′-[γ-^{32}P]triphosphate, tetra(triethylammonium) salt, 2–10 Ci/mmole, [γ-^{32}P]ATP, commercial preparation or prepared as described by Johnson and Walseth (*this volume*).
Adenosine 3′,5′-cyclic monophosphoric acid (cAMP)
Guanosine 3′,5′-cyclic monophosphoric acid (cGMP)
Trichloroacetic acid (TCA)
Ethanol, 95%
Ethyl ether, anhydrous
*Toluene
*2,5-Diphenyloxazole (PPO)
*1,4-bis[2-(5-Phenyloxazolyl)]benzene (POPOP)

2. *Binding assay.* In addition to starred reagents above, the following reagents are required:

Bovine serum albumin (BSA)
[8-^{3}H]adenosine 3′,5′-cyclic monophosphate, ammonium salt, 10–30 Ci/mmole ([^{3}H]cAMP)
[8-^{3}H]guanosine 3′,5′-cyclic monophosphate, ammonium salt, 10–30 Ci/mmole ([^{3}H]cGMP)

IV. PREPARATION OF SOLUTIONS

A. Kinase Assay

1. **Potassium phosphate/DTT buffer (PO_4-DTT) (0.3 M potassium phosphate, pH 6.8; 0.02 M DTT.* Dissolve 40.8 g KH_2PO_4 in water and dilute to 1,000 ml. Adjust pH accurately with an aqueous solution of $K_2HPO_4 \cdot 3\ H_2O$ (68.4 g/1,000 ml). Add 308 mg DTT/100 ml buffer.

2. **Magnesium chloride (0.1 M).* Dissolve 2.03 g $MgCl_2 \cdot 6\ H_2O$ in water and dilute to 100 ml.

3. *Histone (10 mg/ml).* Dissolve 10 mg histone in 1 ml water.

4. *[γ-^{32}P]ATP (5.0 mM).* Dissolve approximately 3 mg ATP in 1 ml water and adjust pH to 6.8 with 1 N KOH. Add 2×10^6 cpm (~0.1 mCi) [γ-^{32}P]ATP/ml. Concentration of solution is accurately determined by absorbance at 259 nm ($E_m = 15.3 \times 10^3$). Add water as needed to bring con-

centration to 5.0 mM. An aliquot (5 μl) is spotted onto a 2.4-cm paper filter disk and counted to determine specific activity.

5. *cAMP- or cGMP-dependent protein kinase (2–40 kinase units U/ml).* One unit of activity equals 1 nmole ^{32}P incorporated in histone H2b per min under standard assay conditions.

6. *TCA (100% w/v).* Dissolve 454 g TCA in 200 ml water. Due to the instability of dilute aqueous solutions, 5 and 10% solutions should be freshly prepared from 100% stock solution.

7. **Scintillation solution.* Dissolve 4.0 g PPO and 50 mg POPOP in 1,000 ml toluene.

B. Binding Assay

In addition to starred solutions above, the following solutions are required:

1. *BSA (10 mg/ml).* Dissolve 100 mg BSA in 10 ml water.

2. *[^{3}H]cAMP or [^{3}H]cGMP (2×10^{-6} M).* Appropriate dilution is made from stock solutions obtained from supplier. Concentration of stock solution is determined by absorbance at 256 ($E_m = 14.5 \times 10^3$) or 252 ($E_m = 13.7 \times 10^3$) nm for cAMP and cGMP, respectively.

3. *cAMP- or cGMP-dependent protein kinase (10–500 binding U/ml).* One unit of activity equals 1 pmole [^{3}H]cyclic nucleotide bound under standard assay conditions.

4. *Potassium phosphate/magnesium chloride buffer (PM) (30 mM potassium phosphate, pH 6.8; 10 mM $MgCl_2$).* Dilute 100 ml 0.3 M PO_4-DTT buffer, pH 6.8, to 1,000 ml. Add 2.0 g $MgCl_2$/1,000 ml buffer.

All aqueous solutions are prepared with glass-distilled water.

V. ASSAY PROCEDURES

A. Cyclic Nucleotide-Dependent Protein Kinase Assays

Pipet into a small, ice-cold test tube (6 × 50 mm) the reagents shown in Table I.

Start the reaction with the addition of the [γ-^{32}P]ATP solution, mix, and transfer test tube to a water bath (30°C) and incubate for 10 min. Stop the reaction by micropipet transfer of 40 μl from the 50-μl reaction mixture onto a numbered (with soft lead pencil) paper filter disk which is supported above a styrofoam board with a pin. The paper disk with pin is immediately transferred to a beaker of ice-cold 10% TCA (5–10 ml/disk) and allowed to stand 15 min. The pin provides a convenient handle and aids in separating the disks during the washing procedure. A series of assays can be conveniently started and stopped at 15-sec intervals. Three additional 15-min washes are repeated in 5% TCA; the second of these performed at 90°C, the others at 0–5°C. Following the TCA wash, the filters are washed once

TABL

Additions

H_2O
PO_4-DTT buffer
Histone (10 mg/m
$MgCl_2$ (0.1 M)
H_2O or cAMP (1C
H_2O or cGMP (1C
Enzyme (2–40 U/
[γ-^{32}P]ATP (5 ×

each in 95% etha
moved, and the di
vials with 1 ml sci

Assays are gene
in the absence of
dependent activity
cAMP or cGMP.
the presence of hi
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zyme, although aut
and cGMP-depend
used or with [γ-^{32}P
dependent protein
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PKI utilizing the
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with histone H2b

In addition to t
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selectively determi
present (Table 3).
tration used. cAMI
[Mg^{++}] (30–50 mM
not affected (15).
tion of protein con
pendent protein kin
dependent protein

TABLE 1. *Reaction mixtures for protein kinase assays*

Additions	Assay	
	Protein kinase A (μl)	Protein kinase G (μl)
H_2O	20	10
PO_4-DTT buffer	5	5
Histone (10 mg/ml)	5	5
$MgCl_2$ (0.1 M)	5	5
H_2O or cAMP (10^{-6} M)	5	15
H_2O or cGMP (10^{-4} M)	—	—
Enzyme (2–40 U/ml)	5	5
[γ-^{32}P]ATP (5 × 10^{-4} M)	5	5

each in 95% ethanol, ethanol-ether (1:1 v/v), and ether. The pins are removed, and the disks are dried under a heat lamp for 5 min, placed in glass vials with 1 ml scintillation solution, and counted.

Assays are generally performed in duplicate, and a blank is determined in the absence of enzyme. To determine the extent of cyclic nucleotide-dependent activity, assays are performed in the absence and presence of cAMP or cGMP. The enzyme-catalyzed reaction must also be dependent on the presence of histone. If crude enzyme preparations are used, phosphate incorporation in the absence of histone is generally due to endogenous substrates. Endogenous incorporation diminishes with purification of the enzyme, although autophosphorylation has been observed with both the cAMP- and cGMP-dependent protein kinases when either high levels of enzyme are used or with [γ-^{32}P]ATP of extremely high specific activity (12,13). cAMP-dependent protein kinase activity is strongly and specifically inhibited by a heat- and acid-stable protein inhibitor (PKI) (14), whereas cGMP-dependent protein kinase activity is not affected by this inhibitor (4). Table 2 provides data obtained with both enzymes in the presence and absence of PKI utilizing the assay procedures outlined above. With these conditions, the enzymic rate is linear up to about 1.5 nmoles of phosphate incorporation with histone H2b as substrate at the saturation level indicated.

In addition to the specificity provided by the PKI, the specificity, and therefore the concentration, of cyclic nucleotide in the assay can be used to selectively determine relative levels of each activity when both enzymes are present (Table 3). Additional specificity is conferred by the Mg^{2+} concentration used. cAMP-dependent protein kinase activity is depressed at higher [Mg^{2+}] (30–50 mM), whereas cGMP-dependent protein kinase activity is not affected (15). Effects of the assay conditions on each enzyme as a function of protein concentration are shown in Fig. 1. Because the cAMP-dependent protein kinase activity is not significantly expressed under cGMP-dependent protein kinase assay conditions, cGMP-dependent protein kinase

TABLE 2. *cAMP- and cGMP-dependent protein kinase activities in the presence and absence of PKI*

Addition[a]	^{32}P incorporation[b]			
	cpm/10 min		pmoles/min	
	−cAMP	+cAMP	−cAMP	+cAMP
Protein kinase A assay				
Histone	190	150	—	—
Histone + PKI	180	170	—	—
Protein kinase A	90	100	—	—
Protein kinase A + PKI	90	120	—	—
Histone + protein kinase A	3,750	19,420	18.8	101.0
Histone + protein kinase A + PKI	1,260	1,610	5.7	7.5
	−cGMP	+cGMP	−cGMP	+cGMP
Protein kinase G assay				
Histone	190	150	—	—
Histone + PKI	190	160	—	—
Protein kinase G	90	90	—	—
Protein kinase G + PKI	80	90	—	—
Histone + protein kinase G	6,960	19,220	35.6	99.8
Histone + protein kinase G + PKI	6,720	20,530	34.3	107.0

[a] The specific activity of protein kinase A and protein kinase G is 61 U/mg and 798 U/mg, respectively. The amount of PKI used was such that complete inhibition of cAMP-dependent protein kinase activity was achieved.
[b] Assays of kinase activities were performed as described in the text with additions as indicated. Specific activity of [γ-^{32}P]ATP is 23.8 cpm/pmole.

$$\text{Conversion factor} = \frac{\text{observed cpm} - \text{blank cpm}}{\text{reaction time} \times \text{specific activity of ATP} \times 0.60} = \text{pmoles/min}$$

activity is readily apparent. Although the activity seen in the cAMP-dependent protein kinase assay can be partially due to cGMP-dependent protein kinase activity, the activity expressed in the presence of PKI should serve to differentiate between them and aid in determining the relative amounts of each enzyme present.

A heat- and acid-stable protein modulator of cGMP-dependent protein kinase activity has also been described (16). This protein can be separated from the cAMP-dependent PKI by chromatography on Sephadex G-100. The modulator protein stimulates cGMP-dependent protein kinase activity when histone is used as substrate. This modulator protein can be used under defined conditions to specifically augment cGMP-dependent protein kinase activity (Fig 2). Current evidence indicates that the effect of modulator protein is limited to histone substrates.

The success of the kinase assay depends largely on the use of endogenous substrate-free enzyme preparations. Crude enzyme preparations may also contain significant levels of ATPase activity, and the addition of fluoride ion

TABLE 3. *cAMP- an*

Enzyme[a]	
Protein kinase A	
Protein kinase G	
Protein kinase A + prc	

[a] The specific activity
respectively.
[b] Specific activity of
[c] 10 mM $MgCl_2$, 0.1 μ
[d] 30 mM $MgCl_2$, 0.1 μ

(40 mM) to the a
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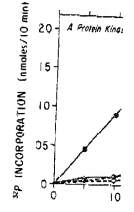

FIG. 1. Specificity of a
lyzed by protein kinase
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observed with protein
kinase G assay conditi
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se activities in the presence and

^{32}P incorporation[b]		
) min	pmoles/min	
+cAMP	−cAMP	+cAMP
150	—	—
170	—	—
100	—	—
120	—	—
19,420	18.8	101.0
1,610	5.7	7.5
+cGMP	−cGMP	+cGMP
150	—	—
160	—	—
90	—	—
90	—	—
19,220	35.6	99.8
20,530	34.3	107.0

nase G is 81 U/mg and 786 U/mg, plete inhibition of cAMP-dependent

bed in the text with additions as lo

$$\frac{\text{cpm}}{\text{of ATP} \times 0.60} = \text{pmoles/min}$$

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y on the use of endogenous me preparations may also the addition of fluoride ion

TABLE 3. *cAMP- and cGMP-dependent protein kinase activities under protein kinase A and protein kinase G assay conditions*

Enzyme[a]	^{32}P incorporation[b] (pmoles/min)			
	Protein kinase A assay[c]		Protein kinase G assay[d]	
	−cAMP	+cAMP	−cGMP	+cGMP
Protein kinase A	19	101	11	20
Protein kinase G	10	46	19	75
Protein kinase A + protein kinase G	35	137	35	104

[a] The specific activity of protein kinase A and protein kinase G is 81 U/mg and 786 U/mg, respectively.
[b] Specific activity of [γ-^{32}P]ATP is 23.8 cpm/pmole
[c] 10 mM $MgCl_2$, 0.1 μM cAMP.
[d] 30 mM $MgCl_2$, 0.1 μM cGMP.

(40 mM) to the assay has been employed to minimize this effect. Kinase activity is also sensitive to inhibition by calcium ion which may be present in tissue extracts. However, chelation by ethyleneglycol-bis(β-aminoethyl-ether)N,N'-tetraacetic acid (0.25 mM) can alleviate this effect. The cyclic nucleotide concentration can be affected by the presence of phosphodiester-

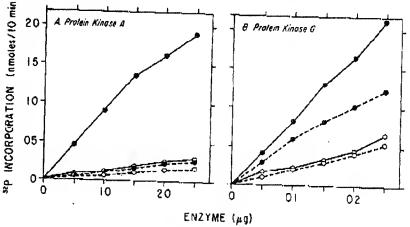

FIG. 1. Specificity of assay conditions on the rate of histone phosphorylation catalyzed by protein kinase A and protein kinase G. Assays are performed as described in the text with amount of enzyme indicated. A: Protein kinase A-catalyzed activity observed with protein kinase A assay conditions (solid lines) and with protein kinase G assay conditions (broken lines). B: Protein kinase G-catalyzed activity observed with protein kinase G assay conditions (solid lines) and with protein kinase A assay conditions (broken lines). ○, minus cyclic nucleotide; ●, plus cyclic nucleotide.

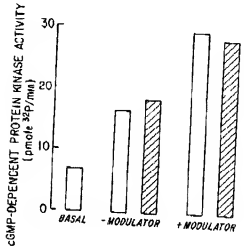

FIG. 2. The effect of modulator protein and PKI on cGMP-dependent protein kinase activity using histone as substrate. Assays contained purified cGMP-dependent protein kinase, cGMP (10 μM), mixed histones (type IIA), and PKI and/or modulator as indicated. Basal activity was determined in the absence of cGMP. Open bars, without PKI; hatched bars, with PKI. (Kindly provided by Dr. John Khoo.)

ase, but this can be inhibited with methylxanthines or other inhibitors of this activity. Theophylline at 1 mM is conveniently used for this purpose.

Product recovery from the assay, at all concentrations used, is another crucial and potential variable which must be assured in order to achieve a reliable assay. TCA precipitation of histone appears adequate, but recovery of more basic substances may require the TCA-tungstate reagent to render them completely insoluble. Despite the solubility characteristics of protamine sulfate and the preparation required for casein, these substrates have been used in specific instances. The specificity of the enzyme used for a particular substrate is an important consideration for optimal activity and when probing for natural substrates of each enzyme. When comparisons of substrates are undertaken, care must be taken to assure that the apparent differences observed are not due to differences in product recoveries.

When [^{32}P]phosphoprotein precipitation is used to determine kinase activity, the phosphoprotein can be isolated from [γ-^{32}P]ATP in a variety of ways. An alternative to the paper filter disk method is to precipitate the product along with a carrier protein such as BSA and filter and wash the product on a glass-fiber disk or Millipore filter. This procedure offers the advantage of utilizing special paper-sensitive reagents in the precipitation of product but is at a disadvantage in the cost of filters and in being a time-consuming procedure. Washing the precipitated product directly in the reac-

tion tube has been e
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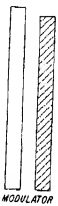

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tion tube has been employed in the past but offers no advantage to the filter disk methods and is time-consuming and cumbersome as well.

When substrate precipitation is not possible, such as with small peptides, ADP production can be monitored with an NADPH-dependent-coupled enzyme system utilizing pyruvate kinase and lactate dehydrogenase. Other methods for measuring kinase activity include the activation and subsequent assay of specific enzymes known to be substrates and whose activity is greatly influenced by the degree of phosphorylation incurred (e.g., phosphorylase kinase, glycogen synthetase, pyruvate kinase, hormone-sensitive lipase). These methods offer an obvious advantage in the determination of specificity of substrate but are undesirable for routine use owing to the complexity of the multistep assay systems. When the activity of interconvertible enzymes is measured, it is desirable to demonstrate reversibility by using phosphatase. An example of such an approach is shown in Fig. 3. Adipose tissue hormone-sensitive lipase, which was activated by incubation with cGMP-dependent protein kinase, cGMP, and ATP·Mg, was inactivated by incubation with

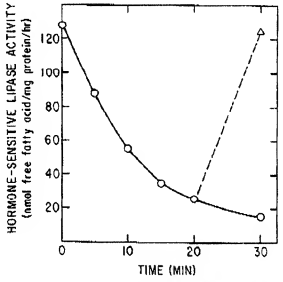

FIG. 3. Reversible deactivation of hormone-sensitive lipase. Chicken adipose tissue lipase was fully activated with cGMP-dependent protein kinase. The activated enzyme was immediately passed through a Sephadex G-50 column to remove ATP, cGMP, and Mg^{2+}. Purified bovine heart phosphorylase phosphatase and 6 mM $MgCl_2$ were added and incubation was carried out at 30°C (○). Reactivation of the deactivated lipase was effected at 20 min by adding ATP, cGMP, and cGMP-dependent protein kinase with further incubation for 10 min at 30°C (△). Basal lipase activity prior to activation was 13 nmoles free fatty acid released/mg protein/hr. From Khoo et al. (11).

purified phosphatase. When cGMP-dependent protein kinase and cofactors were re-added at 20 min, full activation of the hormone-sensitive lipase was again observed.

B. Cyclic Nucleotide Binding Assays

Pipet into a small test tube (6 × 50 mm) the reagents listed in Table 4. Start duplicate reactions with the addition of the enzyme and transfer to a water bath (30°C) and incubate for 5 min. Stop the reaction by transferring the entire mixture with a Pasteur pipet to a Millipore filter reservoir containing 5 ml ice-cold PM buffer. Apply vacuum and filter. Wash the filter 3 times with the same cold buffer to remove unbound labeled nucleotide. A final rinse of the filters with the reservoir removed with cold buffer from a plastic wash bottle will reduce the background level of radioactivity. Filters are dried for 10 min under a heat lamp, placed in counting vials along with 5 ml scintillation fluid, and counted. A blank reaction is routinely performed without enzyme.

In contrast to kinase activity the binding reactions proceed without exogenous magnesium ion; however, some stimulation (~15%) of both enzymes is observed in the presence of 10 mM $MgCl_2$. During early stages of purification, the enzymes demonstrate significant increases in the total binding activity recovered compared to crude extracts, suggesting the presence of inhibitory substances in crude preparations. Besides phosphodiesterase, as mentioned above, crude tissue extracts may contain significant levels of both bound and free cyclic nucleotides. Crude preparations also limit the amount of protein which can be used in the assay owing to the presence of other Millipore binding substances which reduce the number of Millipore retention sites available for cyclic nucleotide binding protein. Therefore, binding should be in the linear range with respect to the amount of protein used. The usual limit is approximately 250 μg of total protein per filter. Binding activity is

TABLE 4. *Reaction mixtures for binding assays*

Additions	Assay	
	Protein kinase A	Protein kinase G
	(μl)	(μl)
H_2O	20	20
PO_4-DTT buffer	5	5
$MgCl_2$ (0.1 M)	5	5
[^{3}H]cAMP (2 × 10^{-4} M)	10	—
[^{3}H]cGMP (2 × 10^{-4} M)	—	10
BSA (10 mg/ml)	5	5
Enzyme (10–500 U/ml)	5	5

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TABLE 5 *Spe*

Addition
None
cAMP
1 × 10^{-7} M
5 × 10^{-7} M
1 × 10^{-6} M
5 × 10^{-6} M
cGMP
1 × 10^{-7} M
5 × 10^{-7} M
1 × 10^{-6} M
5 × 10^{-6} M

[a] Binding was p
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G and 1 × 10^{-7} M
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Assay	
inase A	Protein kinase G
l)	(μl)
0	20
5	5
5	5
0	—
-	10
5	5
5	5

sensitive to sulfhydryl-specific reagents (e.g., *p*-chloromercuribenzoic acid, 5,5′-dithio-bis-2-nitrobenzoic acid), an effect that is overcome by DTT. For routine quantitative assays, excess levels of cyclic nucleotide should be maintained relative to binding sites to ensure maximal saturation of available sites. To ensure saturation, less than 25% of the total cyclic nucleotide added should be bound at equilibrium.

Both cyclic nucleotides bind to the cyclic nucleotide binding sites of both kinases in a competitive manner (17,18). Specificity is such that the K_D for the proper nucleotide exceeds that of the other nucleotide binding 10–100-fold. Because the cyclic nucleotide specificity of each enzyme for binding is similar to that of kinase activation, appropriate concentrations of each cyclic nucleotide can be used to approximate the relative amounts of the two enzymes in tissue extracts (17–19). An example of nucleotide specificity is illustrated in Table 5. Unlabeled cAMP effectively competes with [^{3}H]cAMP binding to protein kinase A but is a much less effective competitor of [^{3}H]cGMP binding to protein kinase G; conversely, unlabeled cGMP specifically inhibits [^{3}H]cGMP binding to protein kinase G. For routine analysis of [^{3}H]cAMP binding to cAMP-dependent protein kinase in impure preparations, 0.1 μM unlabeled cGMP is added to suppress crossover to cGMP-dependent protein kinase; conversely, 0.1 μM unlabeled cAMP is used to suppress crossover of [^{3}H]cGMP to cAMP-dependent protein kinase.

Because the two cyclic nucleotide-dependent protein kinases differ in subunit structure, centrifugation on glycerol or sucrose density gradients can be

TABLE 5 *Specificity of cyclic nucleotide binding to protein kinase A and protein kinase G*

Addition	[^{3}H]cNMP binding[a]			
	Protein kinase A		Protein kinase G	
	cpm	pmol	cpm	pmol
None	13,630	0.673	6,420	0.573
cAMP				
1×10^{-7} M	7,400	0.366	6,080	0.541
5×10^{-7} M	2,680	0.133	3,980	0.355
1×10^{-6} M	1,390	0.069	3,810	0.340
5×10^{-6} M	310	0.015	2,630	0.235
cGMP				
1×10^{-7} M	13,670	0.678	3,460	0.309
5×10^{-7} M	12,290	0.609	1,440	0.129
1×10^{-6} M	13,400	0.663	1,040	0.093
5×10^{-6} M	12,800	0.634	290	0.026

[a] Binding was performed as described in the text with 67 binding U protein kinase A and 1×10^{-7} M [^{3}H]cAMP (90,900 cpm/pmole) or with 57 binding U protein kinase G and 1×10^{-7} M [^{3}H]cGMP (11,200 cpm/pmole) in the presence of unlabeled cyclic nucleotide as indicated.

useful in obtaining separation of binding activities in response to cyclic nucleotide activation and in confirming the relative levels of the two enzyme activities (Fig. 4). In the presence of cAMP, cAMP-dependent protein kinase dissociates into the regulatory cAMP-binding dimer and the catalytic phosphotransferase subunit, whereas the cGMP-dependent protein kinase remains as the undissociated holoenzyme in the presence of cGMP.

The receptor assay can easily be adapted to measure the kinetics of the cyclic nucleotide enzyme interaction under a variety of reaction conditions. The rate of association is determined in standard reaction mixtures equili-

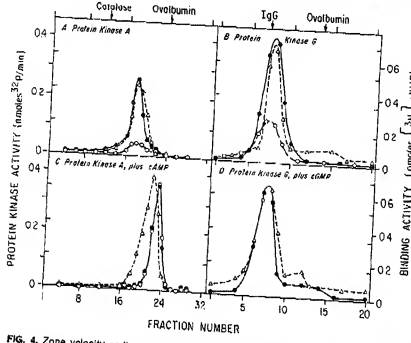

FIG. 4. Zone velocity sedimentation of protein kinase A and protein kinase G. Protein kinase A (1 U/0.2 ml) in the absence of cAMP **(panel A)** and presence of 50 *nM* [^{3}H]cAMP **(panel C)** was layered onto a 5–20% linear sucrose gradient in 50 mM Tris-HCl, pH 7.5, 100 mM KCl, 6 mM 2-mercaptoethanol, 5 mM $MgCl_2$, and centrifuged at 39,000 rpm for 18 hr at 3°C in an SW40 rotor. Protein kinase G (1 U/0.2 ml) in the absence of cGMP **(panel B)** and presence of 2 *nM* [^{3}H]cGMP **(panel D)** was layered onto a 5–30% linear glycerol gradient in 10 mM KH_2PO_4, pH 6.5, and centrifuged at 40,000 rpm for 18 hr at 4°C in an SW56 rotor. Catalase, IgG, and albumin were run concurrently in separate gradients as markers. Fractions were collected from the bottom of the tube and analyzed for protein kinase and cyclic nucleotide binding activities and are expressed as total activity per fraction. **A, C.** protein kinase A activity minus cAMP (○) and plus cAMP (●) and [^{3}H]cAMP binding activity (△). **B, D.** protein kinase G activity minus cGMP (○) and plus cGMP (●) and [^{3}H]cGMP binding activity (△).

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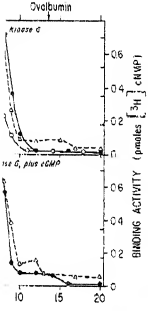

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for protein kinase and cyclic
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brated at the experimental temperature. At time zero, the protein preparation is added to the incubation mixture with stirring; the association reaction is stopped at the desired time by diluting into 5 ml of cold wash solution in the filter reservoir; the bound complex is rapidly isolated by filtering.

The rate of dissociation is determined by allowing the standard binding assay to reach equilibrium with [^{3}H]cyclic nucleotide binding to the regulatory subunits. At time zero, a 1,000-fold excess of unlabeled cyclic nucleotide is added; after additional incubation for varying time periods, the dissociation reaction is stopped by filtering the incubation as in the standard assay. Alternatively, at time zero, the reaction mixture can be diluted 100–1,000-fold.

By performing assays under varying conditions of temperature, pH, and ionic strength, the reaction optima and the thermodynamic parameters for the cyclic nucleotide-protein interaction can be determined (20,21). Other compounds can also be tested for their effect on this interaction. For example, ATP has been found to specifically decrease the affinity of cAMP binding to form I of the cAMP-dependent protein kinase (3).

Competitive protein binding assays for quantitation of cAMP and cGMP have been developed using the appropriate cyclic nucleotide-dependent protein kinase (22–24). In addition, cGMP (25) and cAMP (26) have been quantitated from the extent of activation of their respective cAMP-dependent protein kinases.

ACKNOWLEDGMENTS

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APPENDIX B

PDR MEDICAL DICTIONARY

FIRST EDITION

PDR®

Medical Dictionary

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Indications, adverse reactions and dosage schedules for drugs set forth in this dictionary are provided by the authors. Williams & Wilkins has not independently verified the accuracy of that information and does not make any representation in regard to its accuracy. The reader should review the package information data of the manufacturers of the medications mentioned.

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[illegible] *finger*, hippocratic *nails*, under *nail*. school.

[illegible] (hi-[illegible]-krat'ik). Relating to, described by, or attributed to Hippocrates.

[illegible] **Oath.** An oath demanded of physicians about to [illegible] practice of their profession, the composition of which, [illegible] attributed to Hippocrates of Cos, is probably an [illegible] of the Aesclepiads. It appears in a book of the [illegible] collection as follows:

[illegible] Apollo the physician, by Aesculapius, Hygeia, and [illegible] I take to witness all the gods, all the goddesses, to [illegible] to my ability and my judgment the following [illegible]

[illegible] dear to me as my parents him who taught me this [illegible] in common with him and if necessary to share my [illegible] him; to look upon his children as my own brothers, to [illegible] this art if they so desire without fee or written prom[illegible] to my sons and the sons of the master who taught [illegible] disciples who have enrolled themselves and have [illegible] the rules of the profession, but to these alone, the [illegible] the instruction. I will prescribe regimen for the [illegible] patients according to my ability and my judgment [illegible] harm to anyone. To please no one will I prescribe a [illegible] nor give advice which may cause his death. Nor [illegible] woman a pessary to procure abortion. But I will [illegible] purity of my life and my art. I will not cut for stone, [illegible] in whom the disease is manifest; I will leave [illegible] to be performed by practitioners (specialists in this [illegible] house where I come I will enter only for the good [illegible] keeping myself far from all intentional ill-doing [illegible] and especially from the pleasures of love with [illegible] with men, be they free or slaves. All that may come to [illegible] in the exercise of my profession or outside of my [illegible] in daily commerce with men, which ought not to be [illegible] I will keep secret and will never reveal. If I keep [illegible] faithfully, may I enjoy my life and practice my art, [illegible] by all men and in all times; but if I swerve from it or [illegible] may the reverse be my lot."

[illegible] (hi-pok'rā-tizm). A system of medicine, attrib[illegible] Hippocrates and his disciples, based on the imitation of [illegible] processes in the therapeutic management of disease.

[illegible] (hip'yū-rāt). A salt or ester of hippuric acid.

[illegible] (hi-pyū'rē-ă). The excretion of an abnormally large [illegible] hippuric acid in the urine.

[illegible] **acid** (hi-pyūr'ik). *N*-Benzoylglycine; a detoxifica[illegible] product of benzoate found in the urine of man [illegible] herbivorous animals; used therapeutically in the form [illegible] hippurates of calcium and ammonium). [G. *hippos*, [illegible] urine]

[illegible] (hi-pyūr'i-cās). SYN aminoacylase.

[illegible] Intermittent pupillary dilation and constriction, [illegible] of illumination, convergence, or psychic stimuli. [G. [illegible] from a fancied suggestion of galloping move[illegible]

[illegible] **h.**, dilation of the pupils occurring during forced [illegible] inspiration, and contraction during expiration.

[illegible] Plural of hircus.

[illegible] (her-siz'mŭs). Offensive odor of the axillae. [L. [illegible]

[illegible] pl. **hir·ci** (her'kŭs, her'sī). **1.** The odor of the [illegible] [NA] One of the hairs growing in the axillae. **3.** SYN [illegible] [L. he-goat]

[illegible] Julius, German ophthalmologist, 1843–1925. SEE [illegible]

[illegible] Isador, U.S. dentist, 1881–1965. SEE H.'s *canals*, [illegible]

[illegible] **syn·drome.** See under syndrome.

[illegible] **stain.** See under stain.

[illegible] Harald, Danish physician, 1830–1916. SEE H.'s [illegible]

[illegible] Relating to or characterized by hirsutism. [L. [illegible] shaggy]

hir·su·ti·es (her-sū'tē-ēz). SYN hirsutism. [Mod. L., fr. L. *hirsutus*, shaggy]

hir·sut·ism (her'sū-tizm). Presence of excessive bodily and facial terminal hair, in a male pattern, especially in women; may be present in normal adults as an expression of an ethnic characteristic or may develop in children or adults as the result of androgen excess due to tumors or drugs, or nonandrogenetic drugs. SYN hirsuties, pilosis. [L. *hirsutus*, shaggy]

Apert's h., h. caused by a virilizing disorder of adrenocortical origin.

constitutional h., mild to moderate degree of h. present in an individual exhibiting otherwise normal endocrine and reproductive function.

idiopathic h., h. of uncertain origin in women, who may additionally exhibit menstrual abnormalities and infertility.

hir·tel·lous (hĭr'tĕ-lŭs). Having or resembling fine hairs; term describing the filamentous protein polysaccharide coating of microvilli. SEE glycocalyx. [L. *hirtus*, hairy, shaggy]

hir·u·di·cide (hi-rū'di-sīd). An agent that kills leeches. [L. *hirudo*, leech, + *caedo*, to kill]

hir·u·din (hir'yū-din). An antithrombin substance extracted from the salivary glands of the leech that has the property of preventing coagulation of the blood. [L. *hirudo*, leech]

Hir·u·din·ea (hir'ū-din'ē-ă). The leeches, a class of worms (phylum Annelida) with flat, segmented bodies, a sucker at the posterior end, and often a smaller sucker at the anterior end; they are predatory on invertebrate tissues, or feed on blood and tissue exudates of vertebrates. [L. *hirudo*, leech]

hir·u·di·ni·a·sis (hi-rū-di-nī'ă-sis). A condition resulting from leeches attaching themselves to the skin or being taken into the mouth or nose while drinking. [L. *hirudo*, leech, + G. *-iasis*, condition]

hir·u·din·i·za·tion (hi-rū'di-nī-zā'shŭn). **1.** The process of rendering the blood noncoagulable by the injection of hirudin. **2.** The application of leeches.

Hir·u·do (hi-rū'dō). A genus of leeches (class Hirudinea, family Gnathobdellidae). Species previously used in medicine are: *H. australis*, Australian leech; *H. decora*, American leech; *H. interrupta* or *H. troctina*, a leech of northern Africa; *H. medicinalis*, speckled, Swedish, or German leech, the species previously in most general use; *H. m. officinalis*, a variety of the preceding; *H. provincialis*, the green or Hungarian leech; *H. quinquestriata*, five-striped leech. [L. leech]

His, Wilhelm, Jr., German physician, 1863–1934. SEE H.'s *band*, *bundle*, H. bundle *electrogram*, H.'s *spindle*; Kent-H. *bundle*, H.-Tawara *system*.

His, Wilhelm, Sr., Swiss anatomist and embryologist in Germany, 1831–1904. SEE H.'s *copula*, *line*, *rule*, perivascular *space*; *isthmus* of H.

His- Symbol for histidyl.

-His Symbol for histidino.

His. Symbol for histidine.

Hiss, Philip, U.S. bacteriologist, 1868–1913. SEE H.'s *stain*.

his·ta·mi·nase (his-tam'i-nās). SYN *amine* oxidase (copper-containing).

his·ta·mine (his'tă-mēn). 2-(4-Imidazolyl)ethylamine; a depressor amine derived from histidine by histidine decarboxylase and present in ergot and in animal tissues. It is a powerful stimulant of gastric secretion, a constrictor of bronchial smooth muscle and a vasodilator (capillaries and arterioles) that causes a fall in blood pressure. H., or a substance indistinguishable in action from it, is liberated in the skin as a result of injury. When pricked into the skin in high dilution, it causes the triple response.

h. phosphate, used in the treatment of certain allergies, cephalalgia, and acute multiple sclerosis with varying results; also used to test gastric secretory function, in the diagnosis of pheochromocytoma and in the treatment of Ménière's disease; also available as h. acid phosphate.

his·ta·mine-fast. Indicating the absence of the normal response to histamine, especially in speaking of true gastric anacidity.

his·ta·mi·ne·mia (his'tă-mi-nē'mē-ă). The presence of histamine in the circulating blood. [histamine + G. *haima*, blood]

hi

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Antithrombin action of phosvitin and other phosphate-containing polyanions is mediated by heparin cofactor II

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We have examined the antithrombin effects of various phosphate-containing polyanions (including linear polyphosphates, polynucleotides and the phosphoserine glycoprotein, phosvitin) on the glycosaminoglycan-binding plasma proteinase inhibitors, antithrombin III (ATIII) and heparin cofactor II (HCII). These phosphate-containing polyanions accelerate the HCII-thrombin reaction, as much as 1600-fold in the case of phosvitin. The HCII-thrombin reaction with both phosvitin and polynucleotides appears to follow the ternary complex mechanism. The HCII-thrombin complex is rapidly formed in the presence of these phosphate polyanions (each at 10 μg/ml) when ^{125}I-labeled thrombin is incubated with human plasma (ex vivo). None of these phosphate polyanions accelerate the ATIII-thrombin reaction. Our results suggest that the antithrombotic effect of these phosphate-containing polyanions is mediated by HCII activation and not by ATIII.

Heparin cofactor II; Antithrombin III; Phosphate polyanion

1. INTRODUCTION

Heparin is a glycosaminoglycan that is used therapeutically as an anticoagulant [1]. The antithrombin activity of heparin is effected through interaction with two plasma glycoproteins, antithrombin III (ATIII) and heparin cofactor II (HCII) (for review see [2-6]). ATIII inhibits all of the proteinases involved in intrinsic blood coagulation [6]. The coagulation proteinase specificity of HCII is limited to thrombin [7].

The structure of the heparin (polyanion)-binding sites in ATIII and HCII remain to be fully elucidated. However, the antithrombin action of heparin is attributed in part to its ability to bind both inhibitor (ATIII/HCII) and thrombin to form a ternary complex ([8-10] and references cited therein). Dermatan sulfate also accelerates the HCII-thrombin reaction but it has essentially no effect on the ATIII-thrombin reaction [11-13]. We studied the interaction of various phosphate-containing polyanions with HCII and ATIII to examine further the specificity of the heparin (polyanion)-binding sites of these proteinase inhibitors.

We report here that the antithrombin action of various phosphate-containing polyanions (including linear polyphosphates, polynucleotides and phosvitin, a phosphoserine glycoprotein) is mediated through HCII and not through ATIII. The findings further suggest that these phosphate-containing polyanions are potential therapeutic antithrombotics.

2. EXPERIMENTAL

2.1. *Materials*

HCII, ATIII and thrombin were prepared from human plasma as described previously [14,15] and their purity assessed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Linear polyphosphates, polynucleotides, phosvitin, poly(ethyleneglycol) (M_r = 8000), and salmon protamine sulfate were obtained from Sigma (St. Louis, MO). Dansyl-Glu-Gly-

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Arg chloromethylketone (DEGR) was from Calbiochem (La Jolla, CA). Activated partial thromboplastin time (aPTT) reagents were obtained from Pacific Hemostasis (Ventura, CA). ^{125}I-labeled thrombin (with approx. 3×10^{17} dpm/mol proteinase) was prepared as detailed previously [16]. DEGR-thrombin [10] and lysine-modified HCII [17] were prepared essentially as described previously. The M_r values and extinction coefficients ($ml \cdot mg^{-1} \cdot cm^{-1}$ at 280 nm) were taken as 65 600 and 0.593 for HCII, 56 600 and 0.624 for ATIII and 36 600 and 1.75 for thrombin [5].

2.2. *Assays*

HCII (and ATIII) activity was determined by measuring the rate of thrombin inhibition in the absence and presence of either heparin or the phosphate-containing polyanions in 50 mM triethanolamine-acetate, 100 mM NaCl, 0.1% poly(ethyleneglycol) buffer at pH 8.0 and 25°C with at least a 10-fold molar excess of proteinase inhibitor to thrombin as described previously [5,14,15]. Inhibition rate constants were calculated as detailed [16]. The anticoagulant activity of the phosphate-containing polyanions was measured using an aPTT clotting assay following the manufacturer's procedure.

2.3. *Other determinations and methods*

The kinetic model and association rate equations used in this study assume that the heparin-catalyzed ATIII- or HCII-thrombin reaction is analogous to a bireactant enzyme-catalyzed reaction that follows a random order mechanism as described previously [8]. PAGE was performed in the Laemmli buffer system with 7.5% polyacrylamide gels [18]. Plasma incubation with ^{125}I-labeled thrombin was performed essentially as described previously [12,16]. Extrinsic fluorescence measurements of DEGR-thrombin in the absence and presence of various polyanions were performed as described [10].

3. RESULTS

The effect of linear polyphosphates on thrombin inhibition by HCII and ATIII was investigated. Tripolyphosphate enhanced the rate of thrombin inhibition by HCII, but not by ATIII, in a dose-dependent fashion (fig.1A). The maximal increase in activity (~60-fold) was similar when the nucleotide analog, ATP, was substituted for tripolyphosphate in the reaction (fig.1A). Larger polyphosphate species (with average phosphate chain lengths ranging from 4 to 65) also accelerated the HCII-thrombin reaction maximally more than 800-fold but with no effect on the ATIII-thrombin reaction (fig.1B).

In order to examine the influence of the non-phosphate components of polyphosphate-containing compounds, we compared various polynucleotides in their ability to augment the rate of the HCII-thrombin reaction. The rate of thrombin inhibition by HCII in the presence of synthetic

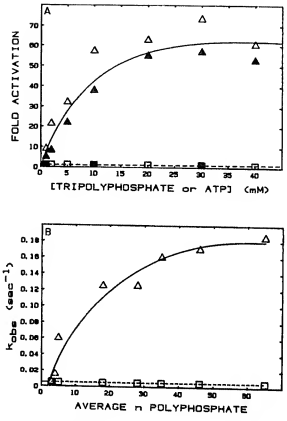

Fig.1. Thrombin inhibition by HCII (△,▲) and ATIII (□,■) in the presence of (A) tripolyphosphate (△,□) or ATP (▲,■) and (B) polyphosphates with average chain lengths ranging from 4 to 65 (each at 1 mM in phosphate) (△,□). The inhibition reaction was performed as described in section 2.

Table 1

Effect of synthetic polynucleotides on the inhibition of thrombin by HCII

Polynucleotide[a]	Rate enhancement (-fold)[b]
Poly(guanylate)	400
Poly(adenylate, guanylate)	430
Poly(inosinate)	160
Poly(guanylate, uridylate)	135
Poly(uridylate)	23
Poly(adenylate)	21
Poly(cytidylate)	12

[a] With the exception of poly(G) which was 10 μg/ml, the polynucleotides were 100 μg/ml in the HCII-thrombin reaction as detailed in section 2

[b] The relative rate of enhancement was compared to the rate constant of thrombin inhibition by HCII in the absence of any polyanion

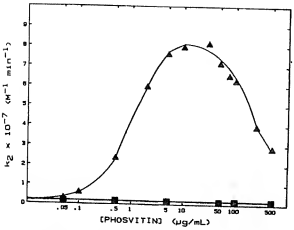

Fig.2. Apparent second order rate constants (k_2) for thrombin inhibition by HCII (▲) and ATIII (■) as a function of phosvitin concentration. Inhibition reaction conditions and rate constant determinations are detailed in section 2.

polynucleotides was significantly greater than the rate measured in the absence of any polyanion (table 1). The maximal effect on HCII-thrombin was exhibited by guanylic acid-containing species. The polynucleotides did not enhance the ATIII-thrombin inhibition rate.

The ability of phosphate groups to accelerate the rate of thrombin inhibition by HCII was further investigated with the phosphoserine-containing glycoprotein, phosvitin. Fig.2 depicts the concentration dependence for stimulation of the HCII-thrombin reaction rate by phosvitin. The rate constant for thrombin inhibition by HCII increased from 5×10^4 $M^{-1} \cdot min^{-1}$ (in the absence of phosvitin) to 8×10^7 $M^{-1} \cdot min^{-1}$ as the phosvitin concentration increased from 0.05 to 30 μg/ml and then decreased as phosvitin was increased above 30 μg/ml. The relationship between poly-(guanylate) (poly(G)) and poly(adenylate, guanylate) concentration and the rate of HCII-thrombin inhibition was similar to that of phosvitin (not shown). As found for the other phosphate-containing polyanions, phosvitin had no effect on the ATIII-thrombin reaction (fig.2).

The kinetic mechanism of the phosvitin-catalyzed HCII-thrombin reaction was evaluated by varying the HCII and thrombin concentration at a fixed phosvitin concentration (fig.3). Saturation kinetics were observed with apparent dissociation constants for HCII-phosvitin and thrombin-phosvitin of 690 and 10 nM, respectively. A similar kinetic analysis for the poly(G)-catalyzed HCII-

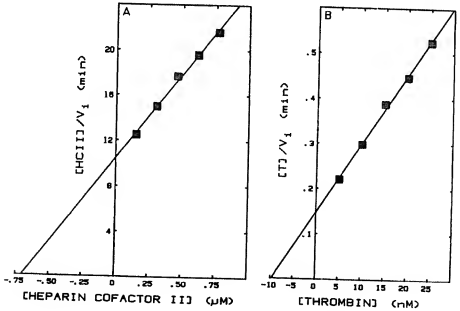

Fig.3. Kinetics of the phosvitin-HCII-thrombin reaction were analyzed by determining the initial rate (v_i) of thrombin (T) inhibition by HCII in the presence of 250 ng/ml phosvitin. (A) Initial T concentration was 5 nM. (B) Initial HCII concentration was 770 nM. Data are plotted as described in section 2.

thrombin reaction yielded apparent dissociation constants for HCII-poly(G) and thrombin-poly(G) of 520 and 15 nM, respectively.

Lysine-modified HCII (phosphopyridoxylated to an extent of 4 mol of reagent incorporated/mol protein) and DEGR-thrombin were used to assess the importance of phosphate-containing polyanion binding to both inhibitor and proteinase during thrombin inhibition. Modified HCII lost >80% of the heparin (and dermatan sulfate) cofactor activity compared to the unmodified proteinase inhibitor. Enhanced thrombin inhibition in the presence of phosphate-containing polyanions [for instance, poly(G), phosvitin and polyphosphate (average chain length of 65)] was greatly reduced (an average of 84%) with lysine-modified HCII. Poly(G) and polyphosphate (average chain length of 65) produced an extrinsic fluorescence signal enhancement (~3-fold) in DEGR-thrombin, indicating that their binding altered the environment of the dansyl moiety in the active site of thrombin.

Calcium and protamine were added to phosvitin and poly(G) to investigate the importance of the polyanion charge on the HCII-thrombin reaction. The phosvitin-catalyzed HCII-thrombin reaction was inhibited more than 97% with rate constants of 6.6×10^6 and $<1.6 \times 10^5\ M^{-1} \cdot min^{-1}$ in the absence and presence of calcium (10 mM), respectively. Protamine (at a 100-fold excess by weight) eliminated >98% of the poly(G) effect on the HCII-thrombin reaction.

The anticoagulant activity of phosvitin, poly(G) and polyphosphate (average chain lengths of 5 and 65) was assessed in plasma. In an aPTT clotting assay, heparin (by weight) is about 100 times more potent as an anticoagulant in plasma than these phosphate-containing polyanions.

The ability of various phosphate-containing polyanions to activate HCII was further investigated in a plasma system. This ex vivo system consisted of incubating ^{125}I-labeled thrombin with plasma and then analyzing the reaction products by SDS-PAGE and autoradiography. As shown in fig.4, incubation of ^{125}I-labeled thrombin with plasma either in the presence of phosvitin, poly(G) or polyphosphate with a chain length of 65 (each at 10 μg/ml) was correlated with incorporation into a complex with HCII. There was no increase in the amount of ^{125}I-labeled thrombin incorporated into a complex with ATIII (fig.4).

Fig.4. Activation of HCII in human plasma was assessed by incubating ^{125}I-labeled thrombin (T) (5 nM), for 10 min at 25°C, in citrated plasma (diluted 1:50) in the presence of phosphate-containing polyanions (each at 10 μg/ml), followed by SDS-PAGE and autoradiography as detailed in section 2. T, purified ATIII-heparin and HCII-dermatan sulfate complexes with T are shown in lanes 1–3, respectively; the plasma system with phosvitin, poly(G) and polyphosphate (average chain length of 65) are in lanes 4–6, respectively.

4. DISCUSSION

In the present study we have shown that various phosphate-containing polyanions greatly enhance the rate of the HCII-catalyzed thrombin inhibition reaction in vitro. Furthermore, the HCII-thrombin complex is rapidly formed when ^{125}I-labeled thrombin is incubated with human plasma (ex vivo) in the presence of either phosvitin, poly(G) or polyphosphate. In all cases, these phosphate-containing polyanions have no effect on the ATIII-catalyzed thrombin reaction in vitro or ex vivo.

HCII is apparently activated by the multiple negative charges of these phosphate polyanions, since the polyanion effect can be negated by complexing the phosphate with calcium or protamine. The effective phosphate polyanions must also possess a specific structure for maximal acceleration of the HCII-thrombin reaction, as shown by the range of rate constants for thrombin inhibition by HCII in the presence of various polynucleotides (also see [19]).

The characteristics of the phosvitin- and polynucleotide-catalyzed HCII-thrombin reaction are similar to those found for HCII and thrombin interactions with heparin or dermatan sulfate [8–10,12,17]. The results with chemically modified HCII illustrate the importance of phosphate polyanion binding to HCII for the catalytic effect during thrombin inhibition. The results with DEGR-thrombin suggest that phosphate polyanion binding to the proteinase is important; this effect is

similar to that reported for heparin-DEGR-thrombin [10]. The rate constant for phosvitin-accelerated thrombin inhibition by HCII increases in a concentration-dependent manner, reaches a maximum, and then decreases as phosvitin concentration is further increased. The shape of the curve implies that phosvitin (and polynucleotides) forms a ternary complex ('template') with binding to both HCII and thrombin. The binding sites for HCII and thrombin probably reside in the phospho-serine-rich core region of phosvitin [20]. This is the first demonstration that a protein or a poly-nucleotide, not a glycosaminoglycan like heparin or dermatan sulfate, can serve as a surface (or template) for thrombin inhibition by a proteinase inhibitor.

Although an in vivo role can be proposed for thrombin inhibition by HCII, the physiological function of this inhibitor remains to be fully understood. Nevertheless, our results support the concept of a new class of antithrombotics that are mediated through HCII and not through ATIII. The common feature of these compounds is a high charge density of phosphate polyanions. Further investigation with these antithrombotics may permit us to evaluate the biological functions of HCII.

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